

Kaempferol induces apoptosis in HepG2 cells via activation of the endoplasmic reticulum stress pathway

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Abstract. Kaempferol is a flavonoid compound that has gained importance due to its antitumor properties; however, the underlying mechanisms remain to be fully understood. The present study aimed to investigate the molecular mechanisms of the antitumor function of kaempferol in HepG2 hepatocellular carcinoma cells. Kaempferol was determined to reduce cell viability, increase lactate dehydrogenase activity and induce apoptosis in a concentration- and time-dependent manner in HepG2 cells. Additionally, kaempferol-induced apoptosis possibly acts via the endoplasmic reticulum (ER) stress pathway, due to the significant increase in the protein expression levels of glucose-regulated protein 78, glucose-regulated protein 94, protein kinase R-like ER kinase, inositol-requiring enzyme 1 α , partial activating transcription factor 6 cleavage, caspase-4, C/EBP homologous protein (CHOP) and cleaved caspase-3. The pro-apoptotic activity of kaempferol was determined to be due to induction of the ER stress-CHOP pathway, as: i) ER stress was blocked by 4-phenyl butyric acid (4-PBA) pretreatment and knockdown of CHOP with small interfering RNA, which resulted in alleviation of kaempferol-induced HepG2 cell apoptosis; and ii) transfection with plasmid overexpressing CHOP reversed the protective effect of 4-PBA in kaempferol-induced HepG2 cells and increased the apoptotic rate. Thus, kaempferol promoted HepG2 cell apoptosis via induction of the ER stress-CHOP signaling pathway. These observations indicate that kaempferol may be used as a

potential chemopreventive treatment strategy for patients with hepatocellular carcinoma.

Introduction

Kaempferol is a flavonoid compound that is found in a variety of vegetables and fruits (1,2); its chemical structure is presented in Fig. 1A. Kaempferol has been used in traditional medicine and has attracted widespread attention due to its various biological functions, including its role as an antioxidant (3), anti-inflammatory (4) and antitumor (5) compound. It has been demonstrated to have number of antitumor effects, including preventing metastasis in oral cancer (6) and inducing apoptosis of colorectal (7), breast (8,9) and prostate cancer (10,11) and leukemia cells (12). A previous study indicated that kaempferol may induce autophagic cell death in SK-HEP-1 human hepatic cancer cells (13). However, to the best of our knowledge, the molecular mechanisms behind the antitumor effects of kaempferol on hepatocellular carcinoma (HCC) remain unknown.

The endoplasmic reticulum (ER) is involved in numerous functions, including protein synthesis, folding and secretion. Disturbances of ER function by stimuli, such as DNA damage, hypoxia, nutritional deprivation and drug toxicity, lead to the ER stress response, which subsequently triggers the unfolded protein response (UPR). As a self-protection mechanism, the UPR reduces protein synthesis and increases the expression of ER molecular chaperones glucose-regulated protein 78 (GRP78) and GRP94 to facilitate the correct folding of proteins (14,15). Protein kinase R-like ER kinase (PERK), inositol-requiring enzyme 1 α (IRE1 α) and activating transcription factor 6 (ATF-6) are the three major transmembrane ER proteins. During ER stress, PERK and IRE1 α are activated and repress protein synthesis via the phosphorylation of the translation initiation factor, eukaryotic initiation factor 2 α . Meanwhile, ATF-6 is transported to the Golgi apparatus, where it is cleaved by Site-1 and Site-2 proteases (14,15). Collectively, these factors activate downstream signaling molecules that trigger a cascade of reactions.

Excessive and prolonged ER stress leads to cellular damage and eventually induces apoptosis. The C/EBP homologous

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protein (CHOP) is the point of convergence for the three aforementioned ER stress transducers (PERK, IRE1 α and ATF-6) and is also the most well-characterized factor in the transition of ER stress to apoptosis (16,17). Proteins in the caspase family are the primary drivers of apoptosis. Human caspase-4 is uniquely located in the ER membrane, where it is specifically activated by ER stress. Similar to caspase-12 in mice, caspase-4 activates caspase-9, in addition to other molecules such as caspase-3, eventually resulting in cell apoptosis (18,19).

A previous study indicated that kaempferol induces apoptosis via the ER stress pathway in U2OS human osteosarcoma cells (20). However, to the best of our knowledge, the importance of ER stress in the antitumor activity of kaempferol in HCC has not been previously elucidated. The present study demonstrated that kaempferol triggers HepG2 apoptosis in a concentration- and time-dependent manner, and indicated that the activation of the ER stress-CHOP pathway is critical for kaempferol-induced apoptosis of HepG2 cells.

Materials and methods

Cells and cell culture. The HepG2 human hepatic cancer cell line was purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were maintained in Dulbecco's modified Eagle's medium (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (Hyclone; GE Healthcare Life Sciences) and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin; Beyotime Institute of Biotechnology, Haimen, China) and incubated at 37°C under a humidified atmosphere of 5% CO₂.

MTT assay for cell viability. HepG2 cells were seeded in 96-well culture plates at a density of 1x10⁴ cells/well. Subsequent to overnight growth, the cells were incubated with 0, 5, 10, 25 50 and 100 μ M kaempferol (Sigma-Aldrich, St. Louis, MO, USA) for 24 h. A separate group of HepG2 cells was treated with 100 μ M kaempferol for time periods of 3, 6, 12 and 24 h. Kaempferol was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich), and the final concentration of DMSO in the culture medium was maintained at <0.1% (v/v). The vehicle (VE) and blank control (BC) groups were established. For the viability assay, at the end of each treatment, 200 μ l culture medium containing 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Amresco LLC, Solon, OH, USA) was added to each well and the mixture was incubated at 37°C for 4 h. The supernatant was removed, formazan crystals were dissolved in 150 μ l DMSO and the absorbance was measured at 570 nm using a microplate reader (Multiskan MK3; Bio-Rad Laboratories, Inc., Hercules, CA, USA). To determine how kaempferol triggers apoptosis via molecular signaling pathways, the following three additional treatment groups were established: i) 4-Phenyl butyric acid (4-PBA; Sigma-Aldrich) pretreatment group, where cells were pretreated with 4-PBA at 1 mM for 30 min; ii) transfection group with CHOP small interfering (si) RNA; and iii) plasmid group with a CHOP overexpressing plasmid (Shanghai GenePharma Co., Ltd., Shanghai, China). HepG2 cells were then exposed to 100 μ M kaempferol for 24 h, and an MTT assay was performed as abovementioned. Cell viability was calculated

as follows: $[(A_{\text{kaempferol treatment group}} - A_{\text{BC}}) / (A_{\text{VE}} - A_{\text{BC}})] \times 100$, where A represents absorbance.

LDH activity assay. A colorimetric lactate dehydrogenase (LDH) activity assay kit (Applygen Technologies, Inc., Beijing, China) was used to quantify the level of LDH released into the supernatant from the damaged cells. This assay was performed subsequent to treatment application. The supernatants from each treatment group were collected and the assay was performed according to the manufacturer's protocol.

Flow cytometric analysis. An Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) double-staining assay (Nanjing KeyGen Biotech, Co., Ltd., Nanjing, China) was used to quantify apoptosis. HepG2 cells were cultivated in 60 mm culture plates for 24 h at 37°C. Following exposure to the indicated treatments, cells were harvested by trypsinization (Sigma-Aldrich), washed with phosphate-buffered saline (PBS), pelleted by centrifugation at 800 x g and 4°C for 5 min, and resuspended in 0.5 ml binding buffer (Nanjing KeyGen Biotech, Co., Ltd.). The cells were incubated with 5 ml annexin V-FITC and 5 ml PI working solution for 15 min at room temperature in the dark. The samples were analyzed on a FACScan flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) using FlowJo software (version 7.6; FlowJo LLC, Ashland, OR, USA). Double staining of cells with annexin V-FITC and PI enabled the identification of different cell populations based on their staining patterns, as follows: Lower left quadrant, live cells (FITC⁻PI⁻); lower right quadrant, early apoptotic cells (FITC⁺PI⁻); upper right quadrant, late apoptotic cells (FITC⁺PI⁺); upper left quadrant, necrotic cells (FITC⁻PI⁺).

CHOP siRNA treatment in vitro. At 24 h prior to transfection, HepG2 cells were prepared in 12-well culture plates. Cells were transfected with 20 μ M human CHOP siRNA and negative control siRNA using Lipofectamine 2000 reagent for 6 h according to the manufacturer's protocol (Shanghai GenePharma Co., Ltd.). CHOP siRNA sequences were designed as follows: Forward (F) 5'-GAGCUCUGAUUGACC GAAUTT-3' and reverse (R) 5'-AUUCGGUCAAUCAGAGCU CTT-3'; control siRNA, F: 5'-UGA GAG UGU CAG CAA UTT CCU-3' and R: 5'-AUC GCU UCA AAG UUA GCG CTT-3'. The transfected cells were then treated with 100 μ M kaempferol for 24 h.

CHOP overexpression plasmid treatment in vitro. HepG2 cells were cultured in 12-well culture plates of 1 ml volumes. CHOP overexpression plasmid (Shanghai GenePharma Co., Ltd.) and the empty vector control plasmid were transfected into HepG2 cells using Lipofectamine 2000 reagent, according to the manufacturer's protocol (Shanghai GenePharma Co., Ltd.). Following a 24 h transfection, HepG2 cells were treated with ER stress inhibitor 4-PBA at 1 mM for 30 min at 37°C, and cultured with 100 μ M kaempferol for an additional 24 h.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. The mRNA levels of target genes were evaluated using RT-qPCR. HepG2 cells were cultured at a concentration of 1x10⁵ cells/well in 12-well

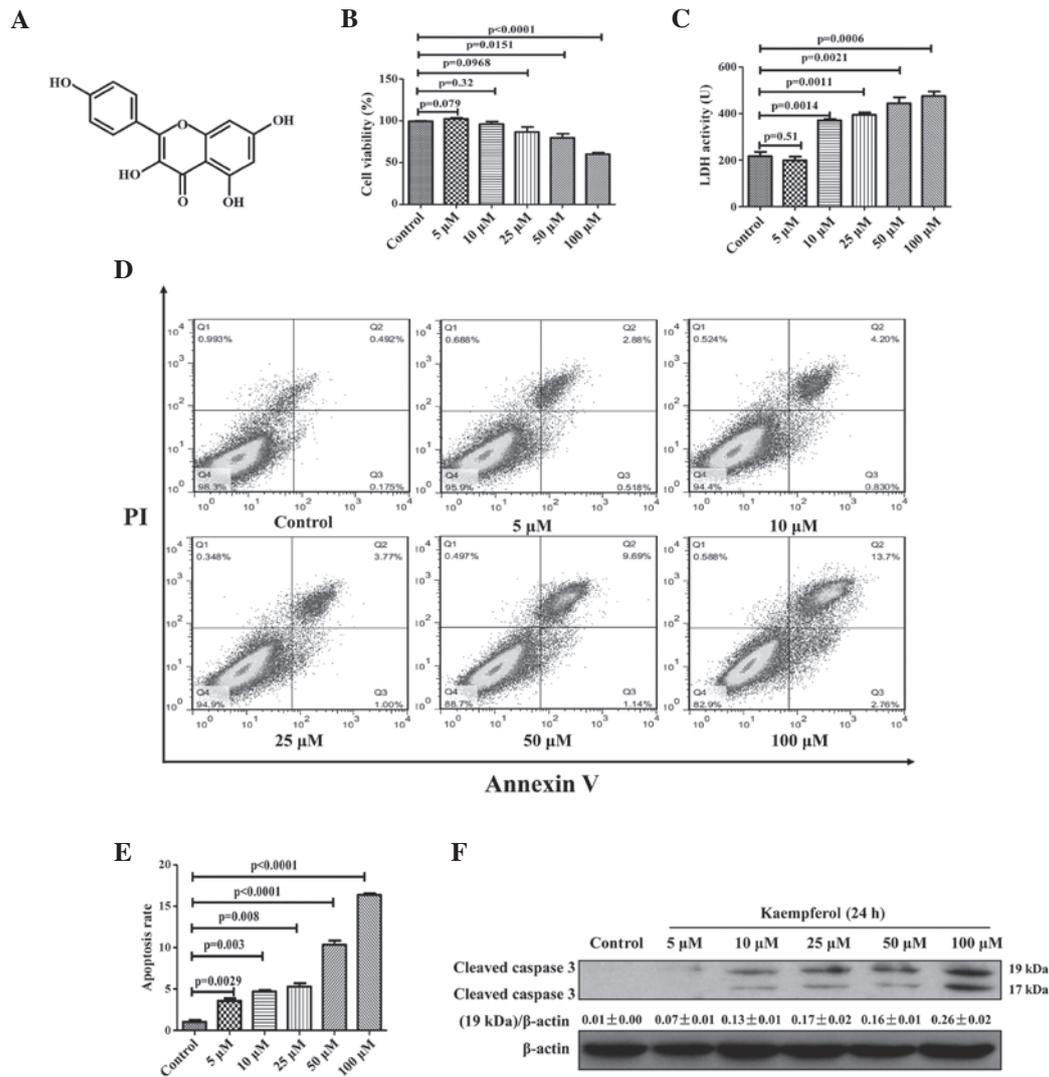


Figure 1. Dose-dependent effects of kaempferol on HepG2 cells. (A) Chemical structure of kaempferol. (B) Cell viability was reduced with higher kaempferol dosage. (C) Cell death was evaluated by determining LDH activity in the supernatant. (D) The apoptotic rate was determined using flow cytometry. (E) Quantification of apoptotic rate. (F) Expression of cleaved caspase-3 and β-actin, was determined using western blotting and quantified by densitometry. All data are expressed as the mean ± standard deviation of at least three independent experiments. LDH, lactate dehydrogenase; PI, propidium iodide.

culture plates. Cells were cultured for 24 h prior to treatments. Total RNA was extracted from HepG2 cells using TRIzol reagent and then reverse-transcribed into cDNA by PrimeScript First Strand cDNA Synthesis kit (Takara Bio, Inc., Otsu, Japan), following the manufacturer's protocol. The hypoxanthine phosphoribosyl transferase (HPRT) gene was selected as an endogenous control. PCR was performed in a reaction mixture (20 μl) containing 4 μl cDNA, 0.4 μl each primer (10 μM), 5.2 μl diethylpyrocarbonate water and 10 μl SYBR Green (Takara Bio, Inc.) using a quantitative PCR instrument (ABI Prism 7500; Applied Biosystems Inc., Waltham, MA, USA). The reaction was processed with an initial 2 min denaturation step at 50°C, followed by 95°C for 5 min, 95°C for 15 sec, and 60°C for 30 sec, for 40 cycles, and then 55°C for 4 sec for 41 cycles. The mRNA levels were calculated using the 2^{-ΔΔCq} method (21). The specific primer sequences for these genes were as follows: HPRT, F 5'-TCA ACGGGGACATAAAAGT-3' and R 5'-TGCATTGTTTA

CCAGTGTCAA-3'; CHOP, F 5'-CCTAGCTTGGCTGAC AGAGG-3' and R 5'-CTGCTCCTTCTCCTTCATGC-3'; GPR78, F 5'-AGTGGTGCCTACCAAGAAGTCTCA-3' and R 5'-TGTCAGGGGTCTTTCACCTTCATA-3'; GPR94, F 5'-ACGTGGTCTGTTTGACGAATATGG-3' and R 5'-TAC ACGGCGCACATAGAGCTTAAT-3'.

Western blot analysis. Subsequent to the indicated treatments, cells were scraped off and washed with ice-cold PBS, and then lysed with radioimmunoprecipitation assay buffer (Sigma-Aldrich) containing a mixture of protease inhibitors. A total of 30 μg of protein from each sample was separated by 12% sodium dodecyl sulfate-polyacrylamide gel (Sigma-Aldrich) electrophoresis at 80 v for 30 min and 120 v for 1 h, and then electrotransferred onto nitrocellulose membranes (Bio-Rad Laboratories, Inc.) using the Bio-Rad Laboratories, Inc. transfer blotting system. The membranes were subsequently incubated with 5% skimmed milk in

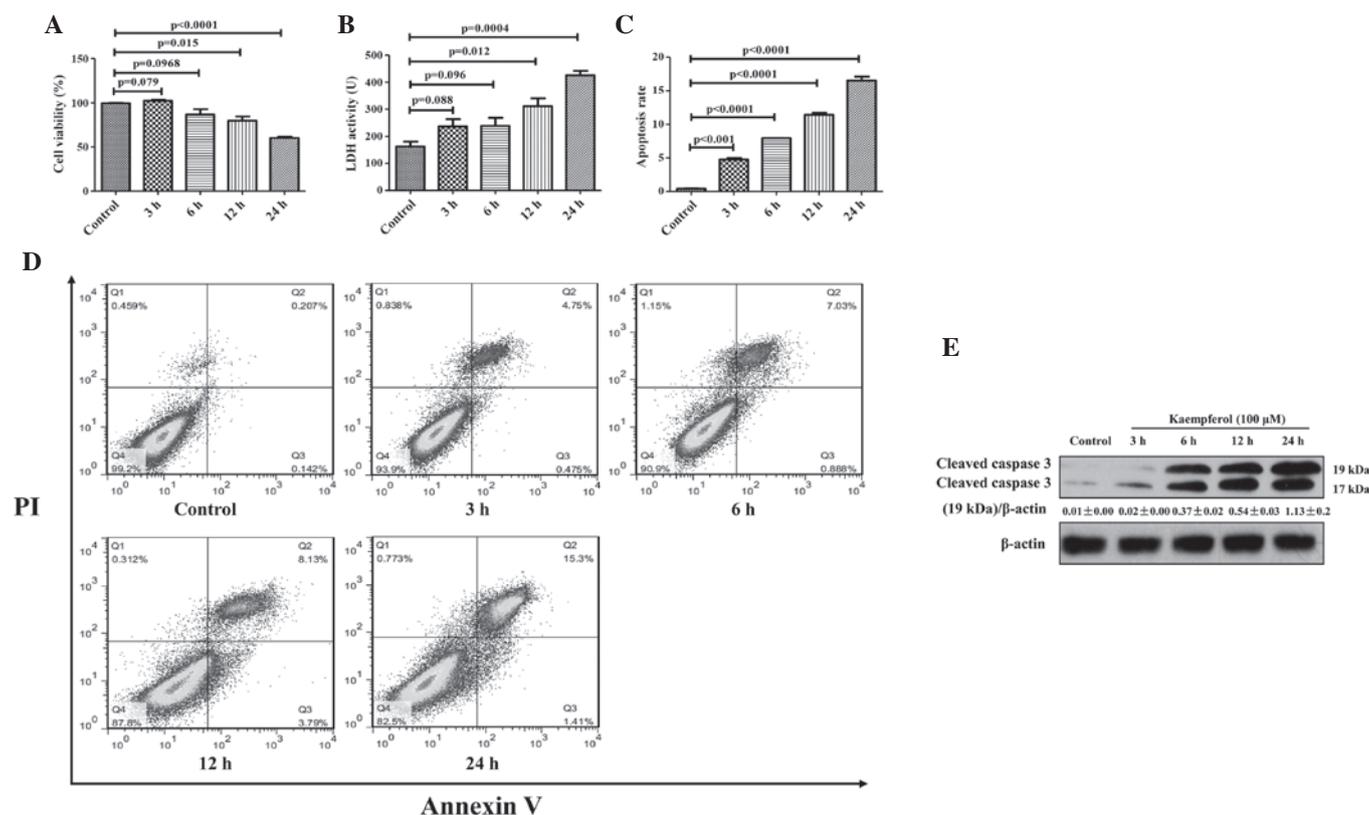


Figure 2. Time-dependent effects of kaempferol on HepG2 cells. (A) Cell viability was reduced with prolonged exposure to kaempferol. (B) Cell death was evaluated by measuring LDH activity in the supernatant. (C) The apoptotic rate was measured using flow cytometry. (D) Quantification of apoptotic rate. (E) Expression of cleaved caspase-3 and β -actin, measured by western blotting and quantified by densitometry. All data are expressed as the mean \pm standard deviation of at least three independent experiments. LDH, lactate dehydrogenase; PI, propidium iodide.

Tris-buffered saline with Tween-20 (Beyotime Institute of Biotechnology) for 1 h to block nonspecific binding and then overnight with the following antibodies: Monoclonal rabbit anti-human GRP78 (1:1000; cat. no. 3177; Cell Signaling Technology, Inc., Danvers, MA, USA), monoclonal rabbit anti-human GRP94 (1:1,000; cat. no. 20292; Cell Signaling Technology, Inc.), monoclonal rabbit anti-human PERK (1:1,000; cat. no. 5683; Cell Signaling Technology, Inc.), monoclonal mouse anti-human partial ATF-6 (1:1,000; cat. no. IMG-273; Imgenex Corporation, San Diego, CA, USA), monoclonal rabbit anti-human IRE1 α (1:1,000; cat. no. 3294; Cell Signaling Technology, Inc.), monoclonal rabbit anti-human caspase-4 (1:1,000; cat. no. 4450; Cell Signaling Technology, Inc.), monoclonal mouse anti-human CHOP (1:1,000; cat. no. 2895; Cell Signaling Technology, Inc.), monoclonal rabbit anti-human cleaved caspase-3 (1:1,000; cat. no. 9664; Cell Signaling Technology, Inc.) and monoclonal rabbit anti-human β -actin (1:1,000; cat. no. 4970; Cell Signaling Technology, Inc.) at 4°C, followed by incubation with monoclonal goat anti-rabbit IgG (1:2,000; cat. no. 14708; Cell Signaling Technology, Inc.) secondary antibody for 1 h at room temperature. Proteins were visualized using an enhanced chemiluminescence commercial kit (Thermo Fisher Scientific, Inc., Rockford, IL, USA). The absorbance of each well containing the protein and reagent was determined at a wavelength of 630 nm using a microplate reader (Multiskan MK3; Thermo Fisher Scientific, Inc.) and a protein concentration standard curve was established to calculate the concentration of each protein sample.

Statistical analysis. Statistical analyses were performed using SPSS statistical software (version 16.0; SPSS, Inc., Chicago, IL, USA). Data are expressed as the mean \pm standard deviation from a minimum of three separate experiments. Statistical comparisons between groups were performed using Student's t-test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Kaempferol induces apoptosis of HepG2 cells in a dose-dependent manner. To investigate the effects of different concentrations of kaempferol on apoptosis, HepG2 cells were treated with 0–100 μ M kaempferol for 24 h. Cell viability was significantly reduced at 100 μ M kaempferol concentration compared with the control group ($P < 0.0001$; Fig. 1B). Additionally, LDH activity ($P = 0.0006$) and the rate of apoptosis significantly increased at 100 μ M kaempferol ($P < 0.0001$; Fig. 1C–E). Western blotting also indicated that kaempferol triggers expression of cleaved caspase-3 (Fig. 1F). The results suggest that kaempferol induces apoptosis in HepG2 cells in a dose-dependent manner.

Kaempferol induces HepG2 apoptosis in a time-dependent manner. To examine whether kaempferol triggers HepG2 cells apoptosis in a time-dependent manner, HepG2 cells were treated with 100 μ M kaempferol for 0, 3, 6, 12 and 24 h. Kaempferol gradually inhibited the proliferation of HepG2 cells

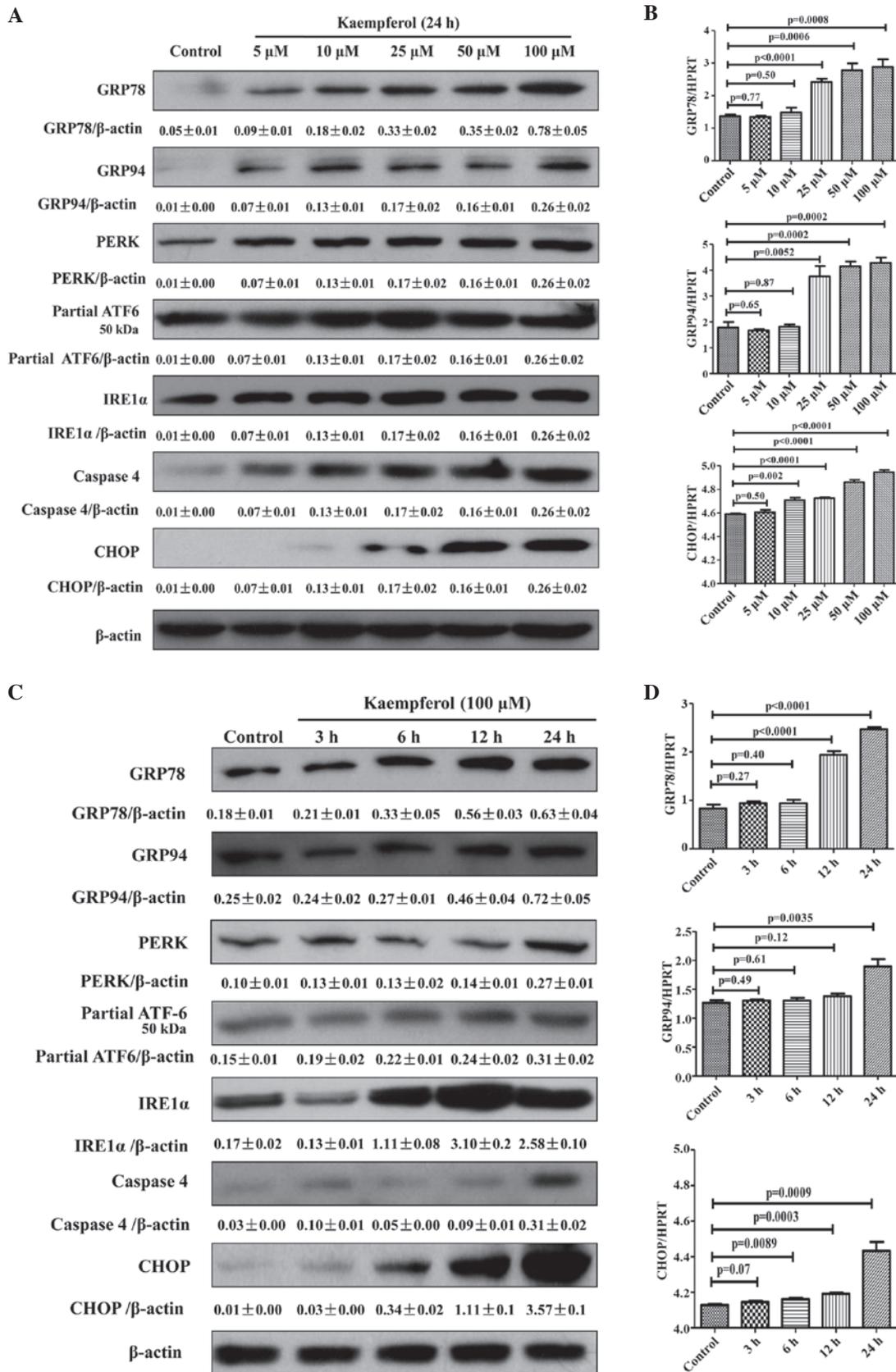


Figure 3. Dose- and time-dependent effects of kaempferol on protein and mRNA levels of endoplasmic reticulum stress markers. (A and B) For dose-dependent effects, HepG2 cells were treated in the presence or absence of different concentrations of kaempferol for 24 h. Western blotting and RT-qPCR were performed with GRP78, GRP94, PERK, partial ATF-6, IRE1 α , caspase-4 and CHOP antibodies. (C and D) For time-dependent effects, HepG2 cells were exposed to 100 μ M kaempferol for different time periods. Western blotting and RT-qPCR were performed with GRP78, GRP94, PERK, partial ATF-6, IRE1 α , caspase-4 and CHOP antibodies. Data are expressed as the mean \pm standard deviation of at least three independent experiments. GRP78, glucose regulated protein 78; GRP94, glucose regulated protein 94; PERK, protein kinase R-like endoplasmic reticulum kinase; ATF-6, activation transcription factor 6; IRE1 α , inositol-requiring enzyme 1 α ; CHOP, C/EBP homologous protein; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; HPRT, hypoxanthine phosphoribosyl transferase.

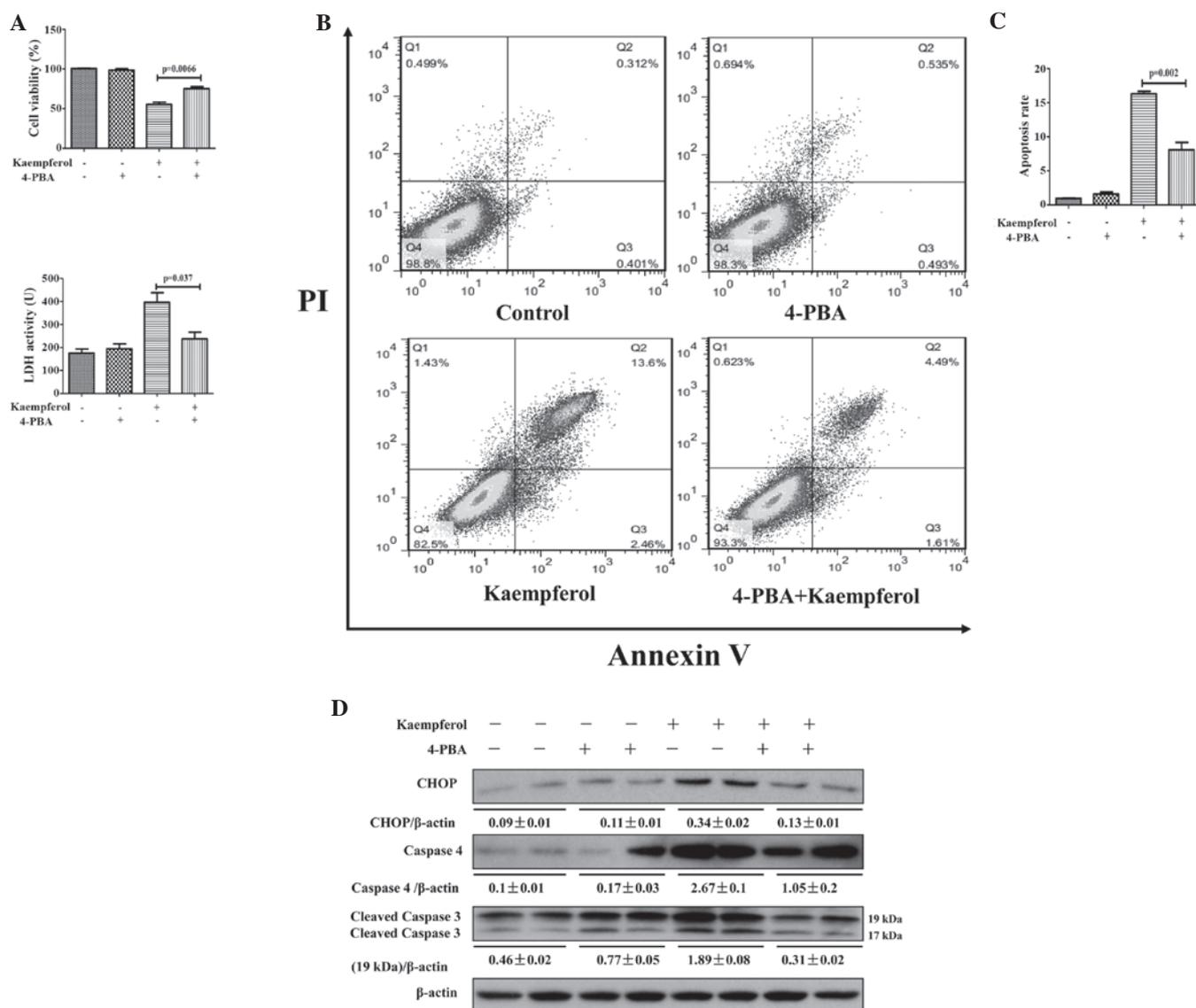


Figure 4. Endoplasmic reticulum stress inhibition by 4-PBA alleviates kaempferol-induced apoptosis in HepG2 cells. (A) Cell viability was higher and LDH activity lower in the cell group pretreated with 4-PBA compared with the kaempferol only group. (B) Apoptotic rate was determined using flow cytometry. (C) Quantification of apoptotic rate. (D) Expression levels of CHOP, caspase-4, cleaved caspase-3 and β -actin were determined using western blotting and quantified by densitometry. Data are expressed as the mean \pm standard deviation of at least three independent experiments. 4-PBA, 4-phenyl butyric acid; LDH, lactate dehydrogenase; CHOP, C/EBP homologous protein; PI, propidium iodide.

and promoted cell death between 12 and 24 h. Following 24 h of 100 μ M kaempferol treatment, cell viability was significantly reduced ($P < 0.0001$; Fig. 2A). There was greater LDH activity ($P < 0.05$; Fig. 2B) and an increased rate of apoptosis ($P < 0.0001$; Fig. 2C and D) in cells exposed to kaempferol for ≥ 3 h compared with the control group. The expression of cleaved caspase-3 was also elevated (Fig. 2E). These data indicate that kaempferol induces apoptosis in HepG2 cells in a time-dependent manner.

Kaempferol triggers ER stress response in a dose- and time-dependent manner. In order to explore the molecular mechanisms of kaempferol-induced apoptosis, the protein and mRNA expression levels of ER stress markers, including GRP78, GRP94, PERK, partial ATF-6, IRE1 α , caspase-4 and CHOP were determined, using western blotting and RT-qPCR, respectively. Kaempferol treatment led to an increase of these

protein and mRNA levels in a dose- and time-dependent manner (Fig. 3A and B). The protein and mRNA levels of GRP78, GRP94 and CHOP were also significantly increased with in a dose-dependent manner (Fig. 3C and D). Therefore, the results indicate that kaempferol-induced apoptosis in HepG2 cells is associated with the induction of the ER stress response.

Inhibition of ER stress alleviates kaempferol-induced HepG2 apoptosis. To confirm the role of the ER stress response as the underlying molecular mechanism of kaempferol-induced apoptosis in HepG2 cells, the cells were pretreated with the ER stress inhibitor 4-PBA to suppress ER stress. Compared with the kaempferol only treatment group, cell viability was significantly higher in the 4-PBA pretreatment group ($P = 0.0066$; Fig. 4A). Additionally, LDH activity

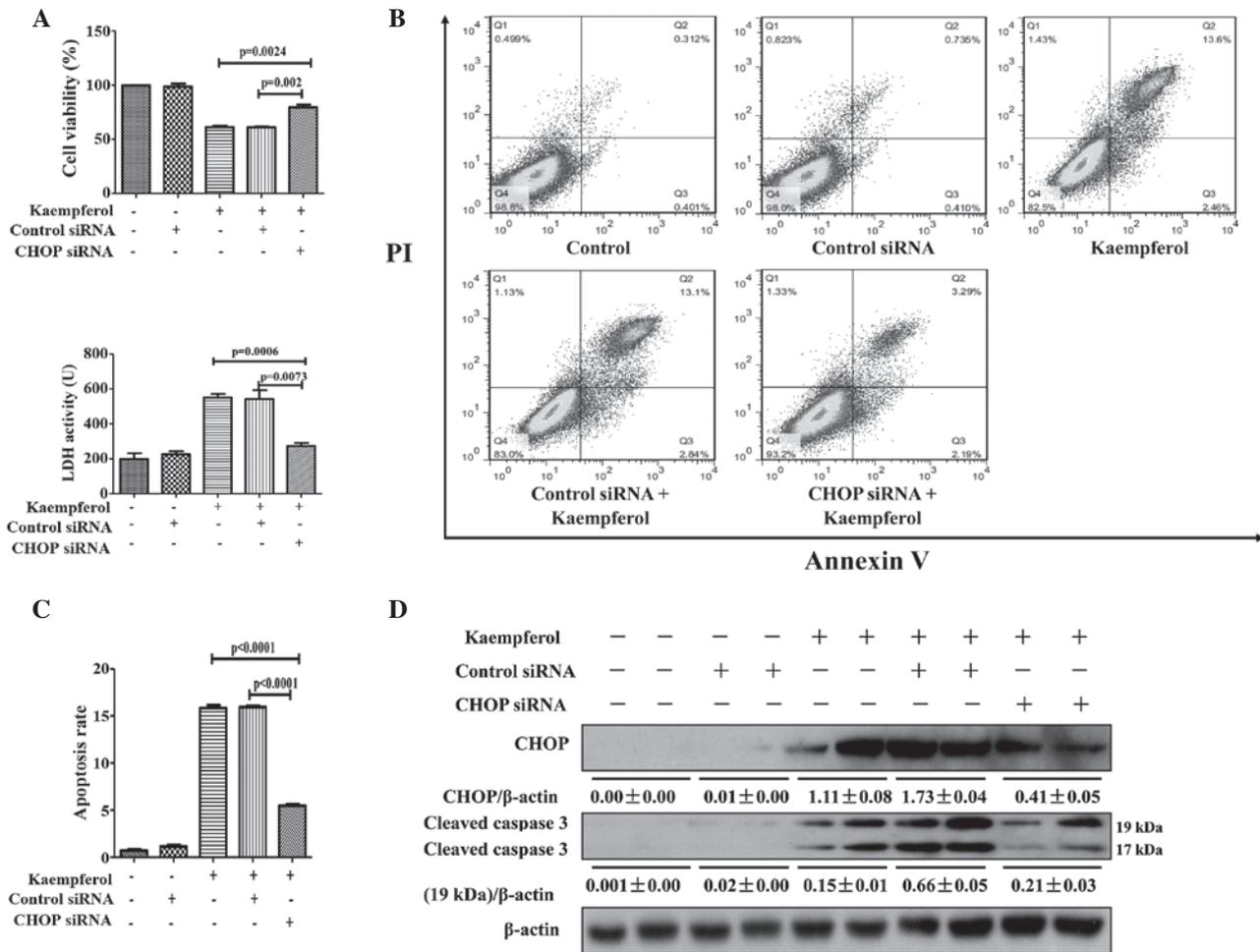


Figure 5. Knockdown of CHOP with siRNA limits kaempferol-induced apoptosis. (A) Kaempferol was found to be less effective in inducing apoptosis in cells transfected with CHOP siRNA. (B) Apoptotic rate was determined using flow cytometry. (C) Quantification of apoptotic rate. (D) Expression levels of CHOP, cleaved caspase-3 and β -actin were determined by western blotting and quantified by densitometry. Data are expressed as the mean \pm standard deviation of at least three independent experiments. CHOP, C/EBP homologous protein; siRNA, small interfering RNA; LDH lactate dehydrogenase; PI, propidium iodide.

and apoptotic rates were lower in the 4-PBA pretreatment group compared with the kaempferol group ($P=0.037$ and $P=0.002$; Fig. 4A-C). Furthermore, the protein expression levels of ER stress markers were evaluated. The protein expression levels of CHOP, caspase-4 and cleaved caspase-3 were observed to be high in the kaempferol treatment group, whereas they were reduced subsequent to the inhibition of ER stress by 4-PBA (Fig. 4D). The results suggest that kaempferol-induced apoptosis in HepG2 cells is mediated by the ER stress response.

Kaempferol triggers ER stress to induce HepG2 apoptosis via the CHOP pathway. CHOP is an extensively-characterized factor in the transition of ER stress to apoptosis (16); therefore, its function in kaempferol-induced apoptosis in HepG2 cells was investigated by limiting its expression. Knockdown of CHOP with siRNA significantly attenuated the reduction of cell viability compared with the kaempferol only treatment group ($P=0.0024$; Fig. 5A). LDH activity ($P=0.0006$), apoptotic rate ($P<0.0001$); and protein expression levels of CHOP and cleaved caspase-3 were markedly reduced in the CHOP siRNA treatment group compared with the kaempferol group (Fig. 5A-D). Following transfection with the

CHOP-overexpressing plasmid, the protein expression levels of CHOP were markedly increased, resulting in a reversal of the protective effect of 4-PBA on kaempferol-induced cell apoptosis (Fig. 6A-D). These results indicate that kaempferol promotes apoptosis of HepG2 cells via the ER stress-CHOP pathway.

Discussion

Kaempferol belongs to the flavonoid family and is found in various foods and traditional Chinese medicines (1,2). As a result of its multiple uses, kaempferol has attracted widespread interest, particularly due to its antitumor properties. The present study indicates that kaempferol inhibits proliferation of HepG2 cells and promotes their apoptosis. The current study demonstrated, for the first time to the best of our knowledge, that kaempferol may induce apoptosis of hepatoma cells via the ER stress-CHOP signaling pathway.

HCC is characterized by high mortality rates and resistance to conventional treatments. Kaempferol has been proposed as a potential agent for HCC treatment due to its antitumor properties (13,22-24). Previous studies have demonstrated that

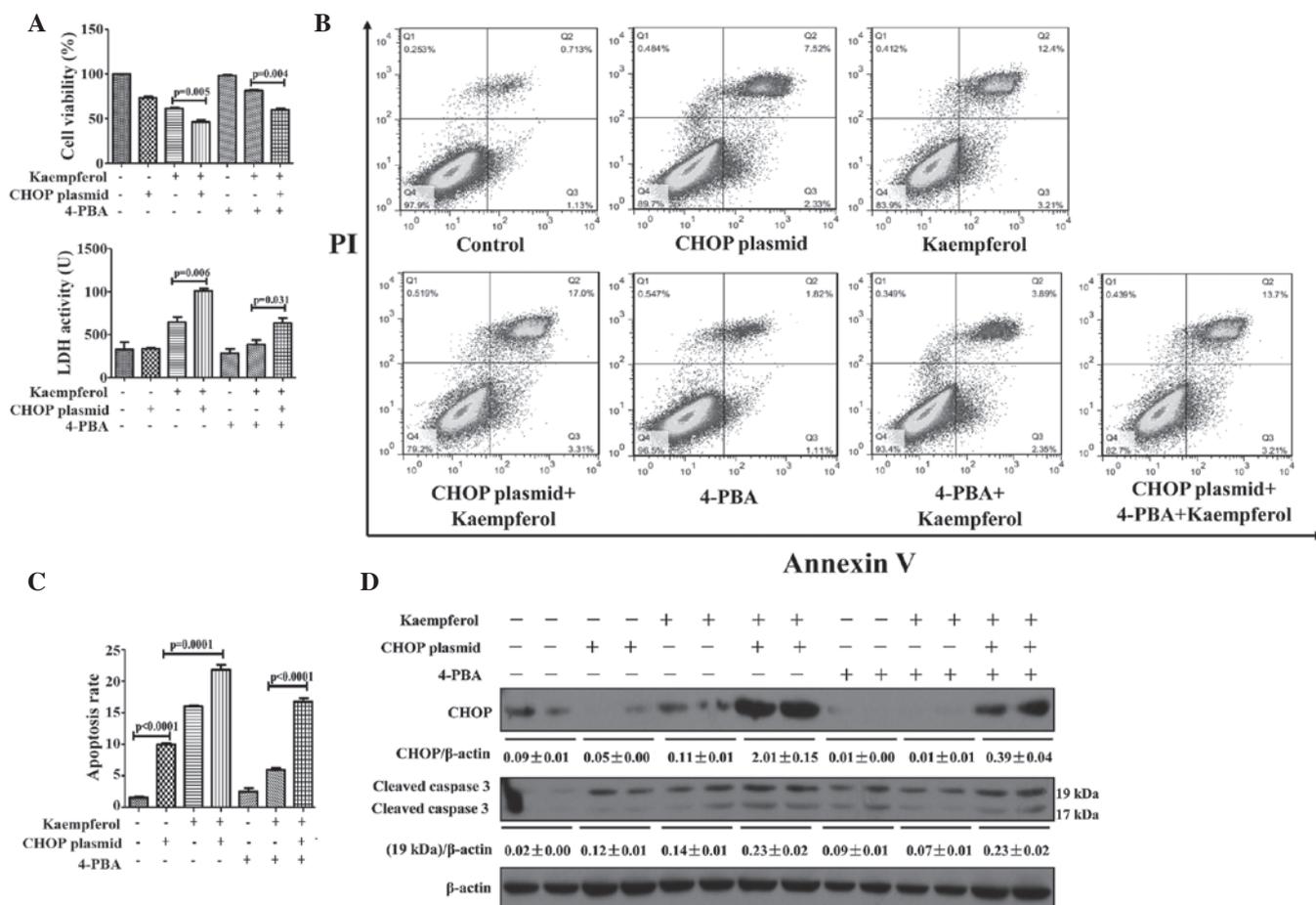


Figure 6. High expression of CHOP by transfection with the plasmid of CHOP reverses the protective effect of 4-PBA on kaempferol-induced HepG2 cell apoptosis. (A) The increased expression of CHOP led to reduced cell viability and higher LDH activity that was not alleviated by 4-PBA. (B) Apoptotic rate was measured using flow cytometry. (C) Quantification of apoptotic rate. (D) Expression of CHOP, cleaved caspase-3 and β -actin was determined by western blotting and quantified by densitometry. Data are expressed as the mean \pm standard deviation of at least three independent experiments. CHOP, C/EBP homologous protein; 4-PBA, 4-phenyl butyric acid; LDH, lactate dehydrogenase; PI, propidium iodide.

kaempferol may inhibit hypoxia-inducible factor-1 activity and induce apoptosis of Huh7 and H4IIE hepatoma cells (22,23). In addition, kaempferol induces autophagic cell death in SK-HEP-1 hepatoma cells through adenosine monophosphate-activated protein kinase and protein kinase B signaling molecules (13). Kaempferol also mediated a reduction in the proliferation rate of Hep3B hepatoma cells (24). HepG2 cells were selected for the current study, as genes of interest in these cells may be easily modified (silencing or overexpression), compared with other hepatoma cells (25). In the present study, the antitumor activity of kaempferol reduced the viability of the tumor cells. Kaempferol damaged HepG2 cells, resulting in intracellular LDH release into the supernatant, which was detected as an increase in LDH activity. These results are in agreement with previous studies (13,22-24), which demonstrated that kaempferol had a distinct inhibitory effect on hepatoma cellular growth. Although the suppressive effect of kaempferol on the growth of hepatoma cells may be evident from these studies, the molecular mechanisms involved remain to be fully elucidated. In current study, the effect of inhibition of proliferation of HepG2 cells was analyzed and ER stress was identified as a novel mechanism in regulating kaempferol-induced apoptosis of HCC.

The ER stress pathway is one of the three classical apoptotic pathways. It is induced in response to numerous conditions of stress (26,27). The inability to properly fold proteins or remove misfolded proteins triggers the UPR to protect cells against ER stress (28,29). The UPR is considered to be a cell survival mechanism; however, if ER stress cannot be alleviated, excessive and prolonged ER stress may activate caspase-4, and then caspase-3, eventually resulting in apoptosis (30). Previous studies have indicated that kaempferol induces ER stress in different cells. Kim *et al* (31) demonstrated that kaempferol protects cells from ischemia/reperfusion-induced cardiac damage through ER stress. Huang *et al* (20) demonstrated that kaempferol induces apoptosis via ER stress and a mitochondrion-dependent pathway in U2OS human osteosarcoma cells. In addition, Chandrika *et al* (32) also determined that kaempferol induces colon cancer cell apoptosis via the ER stress pathway. These results indicate that kaempferol-induced ER stress may be triggered via different molecular pathways in different situations. In the present study, the molecular mechanisms of kaempferol-induced apoptosis via the ER stress pathway were explored. Kaempferol induced apoptosis in HepG2 cells, as confirmed by the apoptosis rate using flow cytometry and by protein expression of cleaved caspase-3

using western blotting. Furthermore, protein and mRNA levels of ER stress markers, GRP78, GRP94, PERK, IRE1 α , partial ATF-6, CHOP and caspase-4, suggested that kaempferol promotes ER stress and induces ER stress-associated apoptosis in HepG2 cells. The current results indicate that promotion of excessive and prolonged ER stress is beneficial for HCC therapy.

In the current study, ER stress inhibition by 4-PBA protected HepG2 cells from kaempferol-induced apoptosis, and the protein expressions of CHOP, caspase-4 and cleaved caspase-3 were markedly reduced in the 4-PBA pretreatment group. The contribution of CHOP to kaempferol-induced apoptosis was investigated by transfecting HepG2 cells with CHOP siRNA. The results of the current study indicated that CHOP siRNA; however not the negative control siRNA, attenuated kaempferol-induced apoptosis. However, apoptosis still occurred following treatment with 4-PBA or CHOP siRNA, suggesting that kaempferol-induced apoptosis was not completely ameliorated. This result suggests that an additional mechanism may be involved in kaempferol-induced apoptosis in HepG2 cells. In addition, to confirm the role of CHOP in kaempferol-induced ER stress, HepG2 cells were transfected with a CHOP-overexpressing plasmid. A marked increase in the apoptosis rate and protein expression of cleaved caspase-3 in the transfected cells subsequent to administration of kaempferol was observed. The overexpression plasmid itself led to a lower apoptosis rate in transfected HepG2 cells, but not the empty vector control plasmid, strongly suggesting that high expression of CHOP promotes kaempferol-induced apoptosis. These data indicate that activation of the ER stress-CHOP pathway is one of the molecular mechanisms of kaempferol-induced HepG2 apoptosis.

In conclusion, the present results have expanded on those of previous studies and confirmed that kaempferol inhibits hepatoma cell growth. Kaempferol-induced apoptosis in HepG2 cells was at least partly mediated by ER stress; however, an additional mechanism involved in kaempferol-induced apoptosis requires further investigation. The current results also strongly suggest that the ER stress-CHOP pathway is important in kaempferol-induced apoptosis. Therefore, kaempferol is a potential therapeutic agent for HCC treatment.

Acknowledgements

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